THE INTRACELLULAR DISTRIBUTION OF 5 - HYDROXYTRYPTAMINE AND HISTAMINE IN A MAST CELL TUMOR

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by

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I. INTRODUCTION

Mast cells have been found in every animal, and in man their total number has been estimated to exceed the weight of the liver (Asboe-Hansen, 1959). They synthesize heparin, histamine and in some species (rat and mouse) 5-hydroxytryptamine (5-HT; serotonin). Although mast cells and their products have been implicated in inflammation and connective tissue repair and allegedly play a role in a number of clinical entities including auto-immune diseases, atherosclerosis, and even schizophrenia, little is actually known of their physiological function or role in disease.

The murine mastocytoma used in this work resembles the normal murine mast cell in that it continues to synthesize heparin, histamine and 5-HT. Aside from learning more about mast cells, the study of these cells in culture allows observations on the mechanisms of amine synthesis and storage in a controlled system with easily determined variables, and hence presents the opportunity to evaluate those factors that influence the level, synthesis, storage, and distribution of amines in cells.

Information obtained on the mechanisms by which mast cells handle 5-HT and histamine almost certainly bears on the mechanism by which other cells handle amines.

II. REVIEW OF THE LITERATURE

Mast cells were originally described in 1877 by Ehrlich, who proposed the name "Mastzellen" (well-fed cells) for the heavily granulated connective tissue cells that stained metachromatically with basic dyes. In 1883 Raudnitz demonstrated the presence of mast cells in man. Mast cells have since been found in practically all species studied, from sponges to primates (Riley, 1959).

A. Morphology and Distribution of Mast Cells (see reviews by Riley, 1959; West, 1959; Asboe-Hansen, 1954)

A characteristic of mammalian mast cells is their variation in size, shape and degree of granulation (also see Simpson and Hayashi, 1960). Usually however, they range from 8 to 20 µ in length and are oval or spindle-shaped; the spindle-shaped cells, presumably younger [called Type I by Riley (1959)], are in close proximity to blood vessels whereas the oval cells,

thought to be mature [called Type II by Riley (1959)], are located in extravascular areas, though the latter cells are often found in perivascular areas. Mast cells contain an oval nucleus, never multilobed like the "blood mast cell" (basophilic leukocyte), and are normally rich in metachromatic granules which range from 0.2 to 1.0 u in diameter; the smaller granules are usually found in the perivascular spindle-shaped cells. Mast cells are derived from mesenchyme, appear late in the development of the embryo, and are most numerous in organs that are substantially developed before birth. Coincident with the development of the localized metachromasia within the mast cell is a diminution in the generalized metachromasia of the connective tissue, although mast cells do appear in the umbilical cord in which generalized metachromasia persists. In the adult they are found wherever loose connective tissue exists, and hence they are numerous in the loose reticular adventitia of blood vessels, the subserous tissue of the peritoneum, pleura and synovial membranes and in all the subcutaneous and submucous tissue. They are sparse in the parenchyma of organs--*i.e.* kidney, liver--but here too their presence is dependent on the amount of loose vascular connective tissue in the organ. Like other species, man lack mast cells in the brain and other neural tissue except for the pineal body, the area postrema and around the vessels of the choroid plexus (see Green, 1962).

The origin of new mast cells in the adult has not been established. It has long been thought that fibroblasts and/or lymphocytes, which have infiltrated the connective tissue spaces, in some way give rise to mast cells since there is little evidence that the normal adult mast cell undergoes mitosis (see Allen, 1961). However, Allen (1961) has recently reported the occurence of mitosis in normal rat mast cells.

Recent electron microscopic studies have firmly established the morphological features of the mast cell. The possibility that the metachromatic granule might be a type of large mitochondria (Riley, 1954; West, 1959), was eliminated by the work of Smith and Lewis (1957) who showed that the large cytoplasmic granules, which are encased in a membrane, are distinct from mitochondria. This work also illustrated the presence of an endoplasmic reticulum in the non-granular-containing cytoplasm. Hagen et al (1959) confirmed these findings on a murine mastocytoma.

That all granular, connective tissue cells that stain metachromatically represent mast cells is questioned by the work of Hibbs et al (1960) and Phillips et al (1960). In particular they have demonstrated that the "younger", spindle-shaped, finely granulated, perivascular mast cell is chromaffin positive as opposed to the "older" oval shaped, extravascular mast cell which is chromaffin negative. Their electron microscopic studies illustrated morphological differences between the

chromaffin positive "mast cell" and the chromaffin negative cell in that the granules of the former were small and homogenous in appearance, whereas the granules of the latter contained a filamentous or thread-like material similar but not identical to the granules described by Hagen et al (1959) and Smith and Lewis (1957). These authors concluded that many of the metachromatic staining granular cells previously considered as mast cells probably represent chromaffin tissue that store catecholamines..

The relationship of the tissue mast cell and "blood mast cell" or basophilic leukocyte, which was also described by Ehrlich, has not been settled. This latter cell resembles the mast cell in its content of both histamine and a heparin-like acidic mucopolysaccharide. Aside from the polylobed nucleus and smaller size of the basophil the two cells are also morphologically similar in that they both contain water soluble, metachromatic granules (see Fredricks and Maloney, 1959; Boselia and Toone, 1961). As Riley (1959) points out the original contention of Ehrlich that the basophilic leukocyte, unlike the mast cell, is of bone marrow origin, and hence related to the neutrophilic leukocyte and eosinophilic leukocyte, has been firmly established. In spite of this fact and other apparent similarities between the two cells no conclusive statement is possible as to whether they are identical or even analogous.

B. Biochemistry of Mast Cells

In 1937 Holmgren and Wilander demonstrated the presence of heparin in mast cells, and Jorpes *et al* extended and confirmed these studies to man (see Riley, 1959). Green and Day (1960) showed that mast cells do not take up preformed heparin but are able to incorporate glucose, glucosamine and sulfate into the mucopolysaccharide, and Silbert and Brown (1961) produced evidence indicating that mast cells will form both uridine-diphosphate-glucosamine and heparin from glucosamine, thus demonstrating the uridine nucleotide in heparin synthesis. Green and Day (1960) also demonstrated that there were at least three different heparins in a murine mastocytoma.

Riley and West, beginning in 1953, and with a number of different studies firmly established that mast cells contain histamine as well as heparin and that most of the histamine in tissues is present in mast cells. Notable exceptions to this are the brain, pyloric mucosa of the stomach and fetal tissues where histamine is present in the absence, or near absence, of mast cells (see Riley, 1959; Kahlson, 1960; and West, 1959).

The presence of 5-HT, as well as histamine, in rat mast cells was demonstrated by Benditt *et al* in 1955. Parratt and West (1957) (also see West, 1959) showed that this was also true for the mouse but not for other species studied, including man. That human mast cells are devoid of 5-HT has been confirmed

by Sjoerdsma et al (1957) and, Ende and Cherniss (1958).

In 1958 Hagen and Lee demonstrated the presence of histidine decarboxylase and 5-hydroxytryptophan (5-HTP) decarboxylase activity in a mouse mastocytoma (see Hagen, 1961), and found these enzymes to be located in the cellular sap of the cell. These decarboxylases were shown to be pyridoxine-dependent, as in normal rat mast cells (Rothschild and Schayer, 1959; Lagunoff and Benditt, 1959). Numerous other studies, including those of Schindler (1958), and Green and Day (1960), have confirmed the fact that mast cells do indeed synthesize histamine (and 5-HT).

In 1961, Green and Day took issue with the widely held view (see West, 1959) that mast cells are unable to take up preformed (exogenous) histamine by demonstrating, unequivocaly, exogenous histamine (and 5-HT) uptake by a murine mastocytoma. These same authors also demonstrated that the exogenous amines turned over at a different rate from the endogenous histamine and 5-HT.

In 1954 Hagen showed that the histamine of dog's liver was present in a large granular fraction which has been prepared by differential centrifugation of liver homogenates; Mota in 1954 showed that this large granular fraction contained mast cell granules (see reviews by Blaschko, 1956; Hagen, 1961). [The dog is the only species whose liver contains a high number of mast cells (Riley, 1959).] Hagen

also demonstrated that these granules did not exhibit histamine activity unless they were suspended in distilled water or exposed to histamine-releasing chemicals such as compound 48/80 or n-octylamine. Subsequent work by Hagen et al (1959), using density gradient centrifugation, resulted in the isolation, from a murine mastocytoma, of granules containing histamine, 5-HT and heparin. These granules were morphologically and biochemically distinct from mitochondria. This intracellular association of histamine, 5-HT, and heparin in mast cells was confirmed by Green and Day (1960). These studies finally established the proposition, based on numerous morphological data, that the heparin, histamine (and 5-HT) were contained in, and in some way bound, to the mast cell granule (see Riley, 1959; West, 1959). The storage of pharmacologically potent substances by a cell in an inert or bound form is not unique to mast cells, for in other tissues acetylcholine, 5-HT, and catecholamines are held in granules. (see Green, 1962).

Much of the discussion on the mechanism of amine-binding in mast cells has revolved about the possible roles played by the granular material. Aside from heparin, mast cell granules have been found to contain significant amounts of lecithin (phosphatidyl choline), and cephalin (phosphatidyl serine, phosphatidyl enthanolamine) (Riley, 1959). Mast cells also contain the sulfatide, cerebroside sulfate (Green

and Robinson, 1960), and the acidic amino acids, taurine and cysteic acid (Green et al, 1961), though it has not been proved that these substances are part of the granule itself.

Although Riley (1959) did not explain the nature of histamine binding in the mast cell granule on the existence of a histamine-heparinate salt, he did feel that heparin, in some way, was important in binding the amine. His conjecture was based in part on studies that showed that toluidine blue, a base, in combining with heparin to produce metachromasia caused a release of histamine from the cell--as if it were displacing the histamine from heparin. Evidence for implicating heparin in binding mast cell amines is also presented by Green (1962). Hence the fact that heparin and histamine are mutually antagonistic in some biological systems indicates that the two substances may form complexes. Also significant are the observations that in several strains of mouse mastocytoma, heparin and amine levels always reflect each other and that heparin, histamine and 5-HT, in different murine mast cell tumors, had the same intracellular distribution (Green and Day, 1960; Hagen et al 1959). Green also sites the fact that a heparin isolated from murine mast cells was distinct from bovine heparin (Green and Day, 1960) in its high affinity for 5-HT and that this might help to explain why mast cells of the mouse, as opposed to those of other species including the cow, contain 5-HT.

Evidence implicating the acidic lipids in amine binding by the mast cell is likewise indirect and incomplete (see Green, 1962; Riley, 1959). Both phospholipids and cerebroside sulfate have been shown to form complexes with amines, though the existence of such complexes *in vivo* has not been proven. There is, however, evidence that phospholipase A (lecithinase A) can cause the release of histamine from mast cells, and Uvnäs (1958) postulated that the histamine releaser, compound 48/80, may operate by activating this phospholipase. Also Green *et al*, (1961) further demonstrated that cysteic acid can form complexes with 5-HT and histamine.

Asboe-Hansen proposed that mast cells secrete connective tissue hyaluronic acid, perhaps by way of a heparin-like intermediate. The evidence for this, however, is in part contradictory (see West, 1959). Further, on the basis of histochemical and enzymatic characterization of substances produced by mast cells and fibroblasts in the human umbilical cord, Moore and Schoenberg (1958) concluded that it was unlikely that the tissue mast cell is important in the synthesis of ground substance polysaccharides.

C. *Function of Mast Cells* (see reviews by Riley, 1955; 1959; West, 1959; Asboe-Hansen, 1954)

Although mast cells are nearly ubiquitous in the body, little is known of their physiological function or their role in disease. There has been much speculation that mast cells play a role in inflammation and repair.

The sequence of mast cell changes occurring in injured tissue has been summarized by Riley (1959). At the onset of the acute inflammatory process--characterized by arteriolar and capillary dilatation, an increase in capillary permeability and an accumulation of edema fluid--the mast cell count is seen to drop markedly. Concomitant with these changes is a diffuse increase in the basophilia and metachromasia of the ground substance. In subsequent stages of the inflammatory process, when new ground substance and fibrils form, mast cell reappear; if this stage of the connective tissue response is protracted, as in chronic inflammation, there is a marked mast cell hyperplasia.

The fact that intradermal histamine injections produces an arteriolar and capillary response identical to that seen in the initial changes of acute inflammation led to the conclusion that a histamine-like substance or histamine itself may be liberated in response to tissue injury. Riley (1959) and West (1959) have shown that substances that release histamine when injected or applied locally result in an increase in capillary permeability and edema. Sheldon and Bauer (1960) convincingly demonstrated that the development of the early stage of the acute inflammatory response produced by cutaneous fungus infections in the rat coincides exactly with a decrease in the number and granulation of local mast cells and that the evanescence of this part of the inflammatory response paralleled

a regranulation and reappearance of mast cells. By depleting the skin of mast cells with compound 48/80 they showed that this phase of the acute inflammatory response was absent or minimal although the later stages of acute inflammation, i.e. mononuclear cell invasion and fibroplasia, did develop. Similarily, the increase in capillary permeability, secondary to antigen-antibody reactions, did not occur in tissues bereft of mast cells (Draper and Smith, 1961). Mast cells have also been shown to be sensitive to various physical stimuli, and hence local edema is produced by stroking the lesions of urticaria pigmentosa, a disease in which mast cells proliferate in the skin (see West, 1959). Heroux (1961) demonstrated that the inflammatory reaction occurring in the ear of rabbits exposed to cold is characterized by a marked decrease in mast cell counts.

Most of the evidence about the participation of mast cells in the synthesis of ground substance is contradictory and incomplete. Riley (1959) proposed that mast cells may store the basic units of hyaluronic acid (glucosamine and glycuronic acids) in the form of heparin and during the dissolution of mast cell, heparin is released and made available for incorporation into new ground substance. Asboe-Hansen (1954) believes that the mast cell secretes hyaluronic acid by way of a heparin-like precursor, and his claim seems to be supported by the concurrent effects of various hormones on connective

tissue and mast cells. Thus, the adrenal corticoids and thyroxine decrease the number and size of mast cells, the level of acid mucopolysaccharides in ground substance, and the S^{35}-sulfate uptake by connective tissue and, in general, impair the reparative and proliferative response of connective tissue (also see Smyth and Gum, 1961). Because of the sensitivity of mast cells to various hormones Asboe-Hansen (1959) has postulated that the mast cell may indeed be the mediator for the effects of various hormones on connective tissue. This conclusion is supported by work showing that adrenal steroids *in vivo* cause a loss of metachromasia from mast cells. (Hill, 1957; Hill and Pospisil, 1960).

However, there are difficulties in accepting the conclusion that mast cells produce ground substance. First, in embryonic tissue metachromatic substance appears before the mast cell (Riley, 1959). Second, in no stage of mast cell development is a compound with the histochemical characteristics of ground substance polysaccharide demonstrable (Moore and Schoenberg, 1958). Third, autoradiograms showed that the S^{35}-sulfate appearing in the ground substance originates from fibroblasts rather than from mast cells (Kennedy, 1960). Finally, some studies have shown that cortisone and adrenocorticotropin had no effect on the number or morphology of mast cells (Boreus, 1961), nor did cortisone effect the uptake of S^{35}-sulfate by mast cells (Green and Day, 1960).

Recently, some investigators have indicated that the mast cell may influence fibroblastic activity. Sheldon and Bauer (1960) and Boyd and Smith (1959) showed delayed fibroblastic proliferation and decreased tensile strength of healed wounds in experimentally produced cutaneous lesions of rats whose mast cells were degranulated or disrupted by means of compound 48/80. In contrast, Kahlson (1960) reported that repeated injections of compound 48/80 greatly increased the tensile strength of healing wounds, an effect that he attributed to increased histidine decarboxylase activity, which was not associated with the presence of mast cells.

Attempts to implicate mast cells in the anti-lipemic effect at heparin have been inconclusive. Although Pomerance (1958) and Sundberg (1955) showed <u>increased</u> levels of mast cells in vessels of patients with coronary and venous thrombosis, Pollack (1957) demonstrated a <u>decrease</u> in the number of mast cells in areas of atheromatous plaques. Though the latter evidence seems to fit well with the anti-lipemic effect of heparin, Watson (1961) could not show any significant alteration in the morphology or number of mast cells in myocardium of rabbits subjected to acute hypercholesterolemia and coronary thrombosis.

Further, mast cells have been implicated in a number of clinical entities including the "collagen" diseases such as periarteritis nodosa and rheumatoid arthritis (Smyth and Gum, 1961),

allergic conditions such as asthma (Salvato, 1959) and benign and malignant nephrosclerosis (Pavone-Macaluso, 1960).

III. SCOPE AND PURPOSE

The ability of the X-1 mastocytoma to grow as a pure preparation of mast cells in culture as well as in solid form in the mouse presented the unique opportunity to study the intracellular distribution of 5-HT and histamine in this cell under both *in vivo* and *in vitro* conditions. Such studies were carried out. Further, since the mast cells in culture take up preformed amines the subcellular distribution of exogenous and endogenous amines was compared. This comparison was especially important, for differences in turnover rates of exogenous and endogenous amines had suggested that two pools for amines exist in these cells (Green and Day, 1962).

Work was also carried out on the subcellular distribution of some of the acidic substances that are present in mast cells, substances that have been implicated in amine-binding. To this end, the intracellular distribution of heparin, cerebroside sulfate, phospholipids, and taurine were determined. Finally

attempts were made to measure ribonucleic acid in the amine-containing particulate material, since this acidic substance forms complexes with amines (Green, 1962); further, a highly acidic protein, perhaps ribonucleoprotein, has been implicated in the binding of catecholamines in the adrenal medulla (Hillarp, 1960).

IV. EXPERIMENTAL METHODS

A. Mast Cells

The mast cells were derived from the Dunn-Potter mouse mastocytoma, P-815 (Dunn and Potter, 1957). A T-line had been developed from the parent tumor and from this line the polyploid X-1 and the diploid X-2 lines were developed (Schindler, Day and Fischer, 1959). All three cell lines were found to maintain their ability to synthesize and store 5-HT, histamine and heparin, and to retain their cytological characteristics after continuous growth in culture and in mice (Green and Day, 1960). X-1 cells were used in this study. This line was maintained as ascitic tumors in DBA/2 and BDF/1 mice. The cells were also carried in culture using techniques and medium already described (Schindler, Day and Fischer, 1959); the medium differed only in the addition of neomycin and in the substitution of magnesium chloride for magnesium sulfate.

For the experimental work the X-1 cells were grown as solid tumors in mice and in culture.

To produce solid tumors BDF/1 mice were injected subcutaneously in the groin with ascitic cells that had been suspended in sterile culture medium. After about 10 days the mice were killed and the tumors harvested by rapid dissection; the connective tissue was removed. The tumors were then weighed and minced with scissors in ice cold 0.3 M sucrose before homogenization. Each tumor weighed about 0.5 g.

To obtain large quantities of cells in culture 2600 to 3200 ml of medium was inoculated with enough culture cells to give an innoculum of 1×10^4 cells/ml. After 72 hours cells were harvested by centrifugation in a Lourdes refrigerated centrifuge. The cells were then washed three times in ice cold 0.9 per cent NaCl, resuspended in a known volume of 0.9 per cent NaCl, an aliquot of which was used for counting in a hemocytometer. Yields ranged from 0.2 to 0.9 g (1×10^6 cells weigh approximately 1 mg).

B. Density Gradient Centrifugation

The procedure for density gradient centrifugation was a modification of that described by Prusoff (1960), Blaschko (1957) and Hagen (1959). Cells were suspended in enough ice cold 0.3 M sucrose to give a 10 per cent concentration and were homogenized with a Teflon homogenizer in the cold. If

culture cells were being used, homogenization was followed by a cell count and if more than 25 per cent of the cells were unbroken homogenization was continued.

The procedure of centrifugation is shown schematically in Fig. 1. The homogenate was centrifuged in the cold in a Lourdes centrifuge at 900 x g for 20 minutes to remove nuclei, cellular debris, and unbroken cells. The precipitate, P-1, was saved for assay and the supernate, S-1, which contained soluble cytsplasmic material (hereafter referred to as soluble material) microsomes, mitochondria, and non-mitochondrial particulate material (granules) was subjected to 10,000 x g for 40 minutes after an aliquot had been removed for assay. This yielded a large granular component, P-2, (mitochondria and granules) and a supernate, S-2, which contained microsomes and soluble material. The pellet of mitochondria and granules was resuspended in a small amount of 0.3 M sucrose and layered over the density gradient. This gradient was prepared about 1 hour before use by carefully pipetting into centrifuge tubes sucrose solutions of decreasing molarity, one over the other. The density gradient that was almost always used contained, from the bottom of the tube up, 2.5 M, 2.0 M, 1.7 M, 1.5 M, 1.2 M and 0.8 M sucrose; the interfaces between these sucrose layers were marked so that the original boundaries could be ascertained after centrifugation. Centrifugation was carried out using the SW-25 head in the Spinco ultracentrifuge for

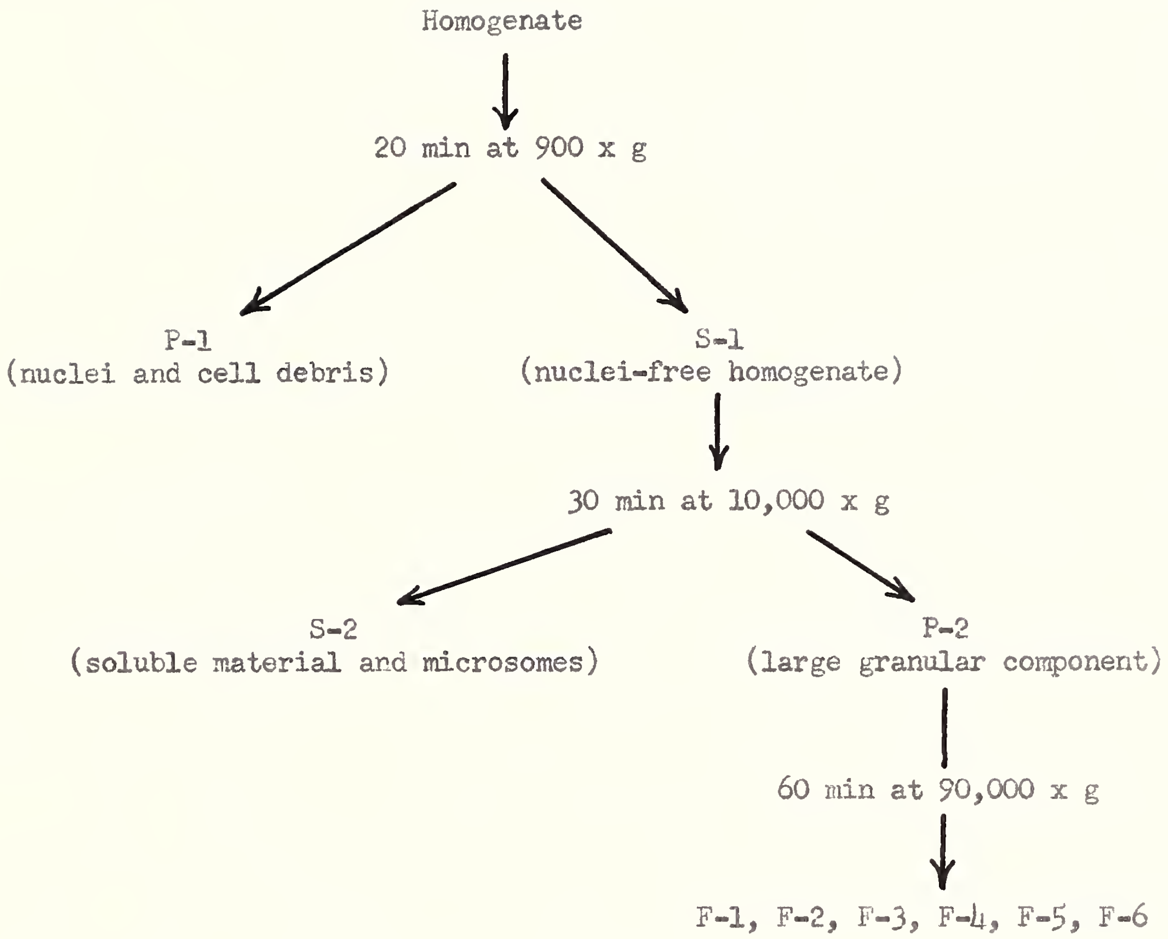


FIGURE 1. SCHEMATIC REPRESENTATION OF THE PROCEDURE FOR DENSITY GRADIENT CENTRIFUGATION.

1 hour at 90,000 x g. At the end of this time the suspension of particulate material had resolved into separate bands at various levels in the centrifuge tube. A diagram was made of the tube, and it was cut at various levels with a Spinco tube slicer to allow separate removal of the different fractions.

C. Analytical Methods

1. Amines

a. Histamine

Histamine was determined in all the subcellular components by the spectrofluorophotometric method described by Shore *et al* (1959). Perchloric acid extracts of the cell fractions were made alkaline with NaOH, saturated with NaCl and shaken with *n*-butanol. The aqueous layer was discarded and the histamine-containing butanol was washed free of any residual histidine with salt saturated NaOH. The histamine was re-extracted into acid by shaking the butanol with heptane and 0.1 N HCl. Histamine was condensed with *o*-phthalaldehyde and fluorescence was read in a Turner fluorometer. Histamine values were calculated by comparing fluorometric readings with those of known amounts of histamine carried through the entire extraction procedure.

b. 5-HT

5-HT assays of the mast cell fractions were carried out by a slightly modified version of the method described by Udenfriend *et al* (1955). Duplicate samples, which had been suspended in water, were saturated with NaCl, and made alkaline with borate buffer. This aqueous extract was then shaken with *n*-butanol. To re-extract the 5-HT into acid the butanol phase was shaken with heptane and 0.1 N HCl. The amount of fluorescence of an aliquot of the acid extract, which had been adjusted to pH 4.0 by the addition of HCl, was determined in a Turner fluorometer. Fluorescence was also determined on known amounts of 5-HT carried through the same procedure.

2. Succinic Oxidase

The location of mitochondria was determined by measuring succinic oxidase in the various cellular fractions. The spectrophotometric method of Slater, with minor modifications, was used. Aliquots of the fractions to be tested were added to a solution containing sodium succinate, KCN, $K_3Fe(CN)_6$ and phosphate buffer at pH 7.2. The change in optical density due to the reduction of $K_3Fe(CN)_6$ was measured at one minute intervals by means of a Beckman DU spectrophotometer. A change of 0.01 in optical density at 400 mμ per 2 minutes represents 100 units of succinic oxidase activity.

3. Heparin

Previous work on these mast cells has shown that they incorporate S^{35}-sulfate into heparin and that the amount of S^{35}-sulfate incorporated into heparin by the cells is related directly to the amount of heparin in the cell (Green and Day, 1960). Heparin in cells that had been grown for the 24 hours before harvesting with 3 μc of S^{35}-sulfate/ml medium was extracted by placing the sample in a dialysis bag with an excess of pancreatin (50-100). The proteolysis-dialysis was carried out against 0.2 M Tris buffer, pH 8.4, for 24 hours and against running tap water for 12 hours. The precipitated material was removed by centrifugation and radioactivity of an aliquot of the supernatant material was measured (see section IV, E).

4. Cerebroside Sulfate

A mixture of chloroform-methanol quantitatively extracts lipids from brain (Lees *et al*, 1959) and other tissue, including mast cells (Green and Robinson, 1960); any sulfur extracted by this means is solely attributed to the sulfolipid, cerebroside sulfate (Lees *et al*, 1959; Green and Robinson, 1960). Green and Robinson (1960) showed that S^{35}-sulfate is incorporated into cerebroside sulfate of mast cells.

Measurements of the amount of cerebroside sulfate in the fractions of mast cells were carried out in the following way. Cells, in culture, were incubated with 3.0 μc of S^{35}-sulfate per ml of medium for 24 hours before they were harvested. After

aliquots of the various subcellular fractions were shaken with 19 volumes of chloroform-methanol (2-1 v/v), the mixture was allowed to stand until the organic and aqueous phases separated. The chloroform-methanol layer was then transferred to a counting vial where it was evaporated to dryness with gentle heat, and radioactivity was measured.

5. Taurine

Taurine was extracted by the method of Awapara (1956) and determined by the method of Hope (1957). The various mast cell fractions were extracted with 80 per cent ethanol, and protein was removed by heat and centrifugation. Enough chloroform was added to yield separate organic and aqueous phases. The aqueous layer was then passed through a Dowex-50-X8, 200-400 mesh, ion-exchange column in the acid form, and the effluent and water-wash were combined and evaporated to dryness *in vacuo*. Samples were resuspended in 1.0 ml. of water and 0.1 ml. aliquots were spotted on Whatman #3 mm filter paper and chromatographed in water-saturated 2,4-lutidine. The amount of taurine was then determined by carrying out a quantitative ninhydrin reaction on the paper. Standards of known taurine, 10 and 20 µg, were run concomitantly.

6. Phospholipid

Phospholipid was determined by measuring total phosphorus (Dryer *et al*, 1957) in chloroform-methanol extracts of the cell

fractions (see Lees et al, 1959). Aliquots of the fractions were extracted with trichloracetic acid, centrifuged and the supernatant was discarded. After the chloroform-methanol solution had been shaken with the precipitate, it was transferred to digestion flasks and evaporated to dryness with gentle heat. The lipid residue was digested with sulfuric acid and hydrogen peroxide. Excess peroxides were destroyed by the addition of 5 per cent urea and any polyphosphates that had been formed during the procedure were hydrolized by boiling the reaction mixture for a few minutes. The contents of the flask were quantitatively transferred to 10 ml graduate cylinders. The addition of ammonium-molybdate and N-phenyl-p-phenylenediamine (i.e. semidine) produced a blue color, the intensity of which was read in a Beckman DU spectrophotometer at 345 mμ. Phosphate standards, 0.5 and 1.0 μmoles of phosphate, were carried through the same procedure.

7. Ribonucleic Acid (RNA)

RNA was measured by the method of Schneider (1957). Samples were mixed with cold trichloracetic acid, centrifuged and the supernatant material was discarded. After the precipitate was made lipid-free by extraction with chloroform-methanol (see section IV, C, 4), it was dissolved in potassium hydroxide and heated to hydrolize the RNA. Protein and desoxyribonucleic acid were precipitated from solution by acidification with HCl and trichloracetic acid. RNA was

determined by analysis of the supernate for pentose by means of the orcinol reaction (Ashwell, 1957).

Alternatively, RNA was extracted with phenol (Kirby, 1956) and measured with a spectrophotometer.

D. Exogenous Amines

Cells, in culture, were incubated with either C^{14}-histamine (*i.e.* histamine-2-ring-C^{14}) or C^{14}5-HT (*i.e.* 5-hydroxy-3-(β-amineoethyl-β-C^{14})) for 24 hours before harvesting. The specific activity of C^{14}-histamine was 1.48 mc/mmole, that of C^{14}-5-HT 6.36 mc/mmole. Cellular fractions were prepared in the usual way (section IV, B), and samples, in duplicate, were carried through the extraction procedures described under amine determination (section IV, C). Aliquots were removed from the final acid-extract for the measurement of radioactivity (section IV, E) and for the determination of histamine and 5-HT.

E. Measurement of Radioactivity

An aliquot, 0.2 ml, of the extract was added to 10 ml of a mixture of 8 g of diphenyloxazole, 100 mg POPOP, 2 l toluene and 1 l absolute ethyl alcohol. Radioactivity was measured in a Tri-Carb liquid scintillation spectrometer.

V. EXPERIMENTAL RESULTS

A. Intracellular Distribution of Endogenous Amines

1. Solid Tumors

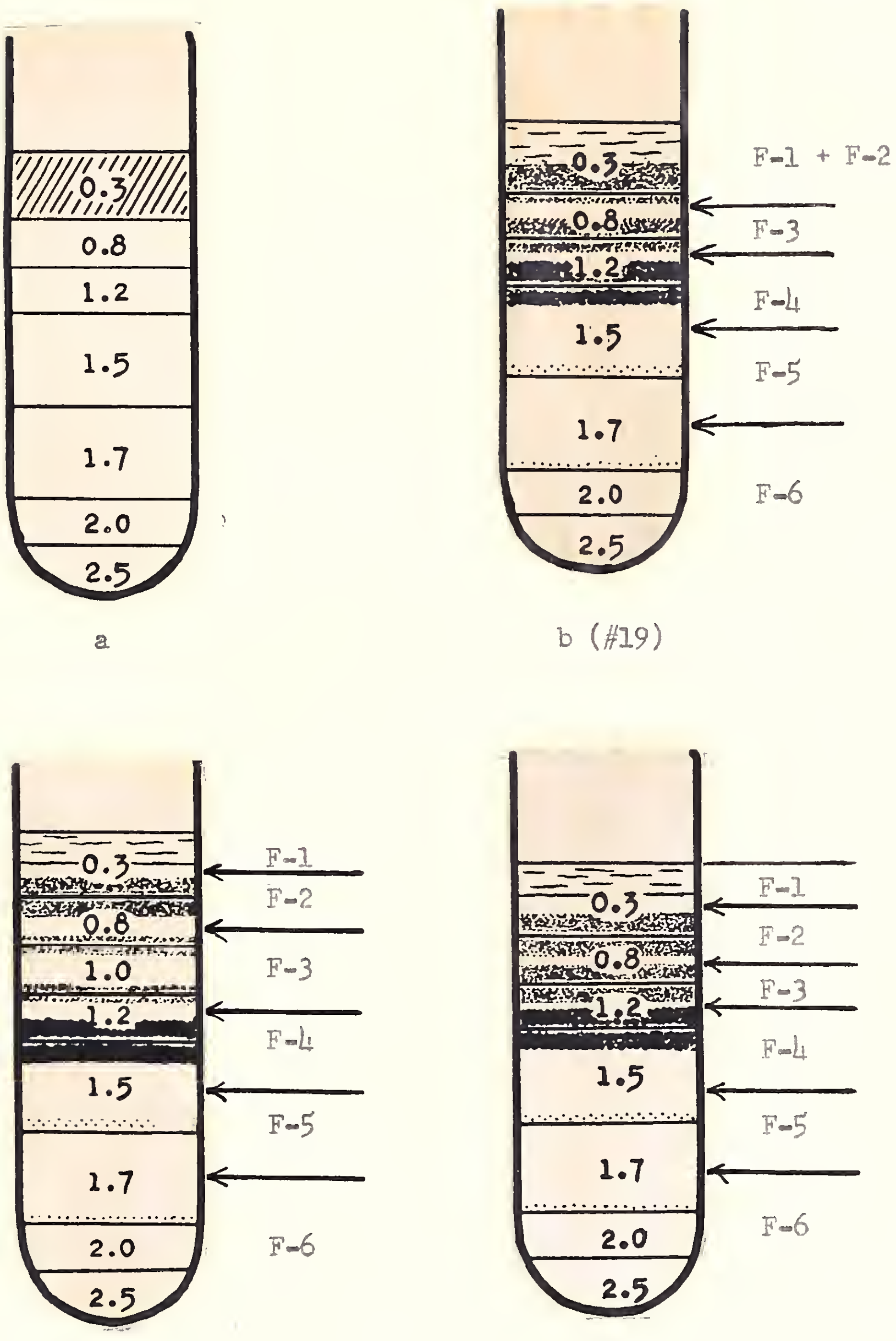
Table 1 shows the distribution of amines and succinic oxidase among the nuclei-free homogenate (S-1), the large granular component (P-2), and S-2 which contains both soluble material and microsomes. At least 60 per cent or more of the amines did not sediment with the large granular component, remaining in the S-2 fraction. This distribution contrasted with that of succinic oxidase the bulk of which was found in the large granular component. These findings indicate that the amine-containing particulate material is less dense than the mitochondria.

Fig. 2 illustrates the appearance of density gradients before and after centrifugation. As shown in Fig. 2a the large granular component, which was resuspended in 3-4 ml of 0.3 M sucrose, has been placed on the density gradient without disturbing the sucrose layers below. Examination of the gradients

exp.	µg amines per mg cell		S-1 (µg or units)	Recovery in S-2 + P-2 (per cent)	S-2 (µg or units)	S-2 (per cent)	P-2* (µg or units)	P-2* (per cent)
19	0.18	5-HT	223.6	56.5	126.0	66.6	63.4	33.4
23	0.012	Histamine	18.0	100.6	10.8	51.7	9.5	49.3
		SO	18,000	87.1	4500	26.8	11,235	73.2
24	0.26	5-HT	403.2	114.4	275.4	60.0	185.8	40.0
	0.28	Histamine	526.2	76.6	265.2	66.0	137.6	34.0
		SO	22,400	111.2	10,200	41.0	14,715	59.0

*The values shown under P-2 are derived by summing the amounts present in the density gradient.

TABLE 1. THE DISTRIBUTION OF AMINES AND SUCCINIC OXIDASE (SO) IN S-1 (NUCLEI-FREE HOMOGENATE), S-2 (SOLUBLE MATERIAL AND MICROSOMES), AND P-2 (LARGE GRANULAR COMPONENT) OF THE X-1 SOLID MASTOCYTOMA.



a

b (#19)

c (#23)

d (#24)

FIGURE 2. DENSITY GRADIENT CENTRIFUGATION OF THE X-1 SOLID MASTOCYTOMA. a) density gradient before centrifugation; b, c & d density gradients after centrifugation. Arrows on the right indicate where tube has been cut. Dark horizontal lines indicate the interfaces between the various sucrose layers.

after centrifugation (Fig. 2b, c, and d) revealed that a distinct, dark band, F-4, consistently appeared at the interface between 1.2 M and 1.5 M sucrose; less distinct, smaller bands, F-5 and F-6, always appeared at the 1.5 - 1.7 M and 1.7 - 2.0 M interfaces. In the experiment shown in Fig. 2b, the least dense fractions, F-1 and F-2, were not as cleanly separated as they were in the accompanying experiments due to a plethora of granular material.

In the tube shown in Fig. 2c the interposition of 1.0 M sucrose between the 0.8 M and 1.2 M sucrose layers separated the single band (F-3) which was seen at the 0.8 - 1.2 M interface in Fig. 2b and 2d. This split occurred only when 1.0 M sucrose was included in the gradient and probably indicates that some of the particulate material that makes up F-3 is very close in density to 1.0 M sucrose. Table 2 clearly shows that the amine-containing particles were less dense than the mitochondria. That the material in the F-4 layer was consistently the richest in succinic oxidase activity established this band as the mitochondria. A total of only 5 to 15 per cent of the 5-HT and histamine was found in the two hazy bands below this fraction, whereas 60 per cent of the amines were distributed, about equally, in the layers of particulate material (F-1, F-2, F-3) that were distinguishable above the mitochondria. The location of the remaining 20 to 30 per cent of the amines in F-4 resulted in a full 80 per cent or more of the total 5-HT and histamine being situated in particulate material as dense as or less

		per cent	
Fractions	exp. 19	exp. 23	exp. 24
F-1			
5-HT	35.0*	----	21.6
Histamine	----	20.9	30.9
SO	----	0.0	3.9
F-2			
5-HT	**	----	18.8
Histamine	----	27.2	19.8
SO	**	7.3	8.1
F-3			
5-HT	28.5	----	19.4
Histamine	----	14.6	16.5
SO	----	14.6	24.4
F-4			
5-HT	30.3	----	18.3
Histamine	----	23.0	23.4
SO	----	57.4	46.2
F-5			
5-HT	0.0	----	9.6
Histamine	----	3.9	3.7
SO	----	12.8	8.6
F-6			
5-HT	6.3	----	12.3
Histamine	----	10.0	5.6
SO	----	7.8	8.5

*Combined with F-2

**Combined with F-1

TABLE 2. THE DISTRIBUTION OF 5-HT, HISTAMINE AND SUCCINIC OXIDASE (SO) IN FRACTIONS AFTER DENSITY GRADIENT CENTRIFUGATION OF THE SOLID MASTOCYTOMA

than the mitochondria. That the ratio of succinic oxidase activity to amine levels in the various fractions was not constant but increased suddenly as F-4 was approached (where it was maximum), indicates that amine-containing particles differed from mitochondria.

It should also be noted that the non-sedimenting material, F-1, differed in gross appearance from the other particulate layers in that it appeared milky and homogeneous in contrast to the remaining fractions which were tan and somewhat granular.

The data in Table 2 also indicate that the distribution of 5-HT and histamine are approximately the same and that this distribution was the same regardless of the total amine level in the cell (see Table 1).

2. Cells in Culture

Table 3 shows an experiment in which amines were measured in all of the major cellular components in cells from culture. In contrast to the experiments on solid tumors (see Table 1) the great majority of 5-HT sedimented with the large granular component.

The distribution of particulate material and endogenous amines in the density gradients obtained with culture cells is illustrated in Fig. 3. The distinct opaque layer, F-3, which consistently appeared in the solid tumor gradients at the 1.2 - 1.5 M interface was evident here. However, in contrast

exp.	µg amines per mg cell		S-1	Recovery in S-2 + P-2	S-2		P-2*	
			(µg or units)	(per cent)	(µg or units)	(per cent)	(µg or units)	(per cent)
37	0.23	5-HT	63.8	52.6	2.70	6.0	31.0	94.0

*The value shown under P-2 is derived by summing the amounts present in the density gradient.

TABLE 3. THE DISTRIBUTION OF 5-HT IN S-1 (NUCLIE-FREE HOMOGENATE), S-2 (SOLUBLE MATERIAL AND MICROSOMES), AND P-2 (LARGE GRANULAR COMPONENT) OF CELLS IN CULTURE

	exp. 37 (per cent)	exp. 45 (per cent)	
	5-HT	Histamine	SO
F-1	7.7	17.8	0.0
F-2	0.0	0.0	14.9
F-3	49.3	43.4	60.5
F-4	42.9	38.8	24.5
F-5	0.0	0.0	0.0

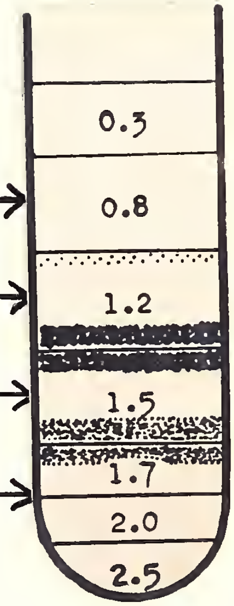
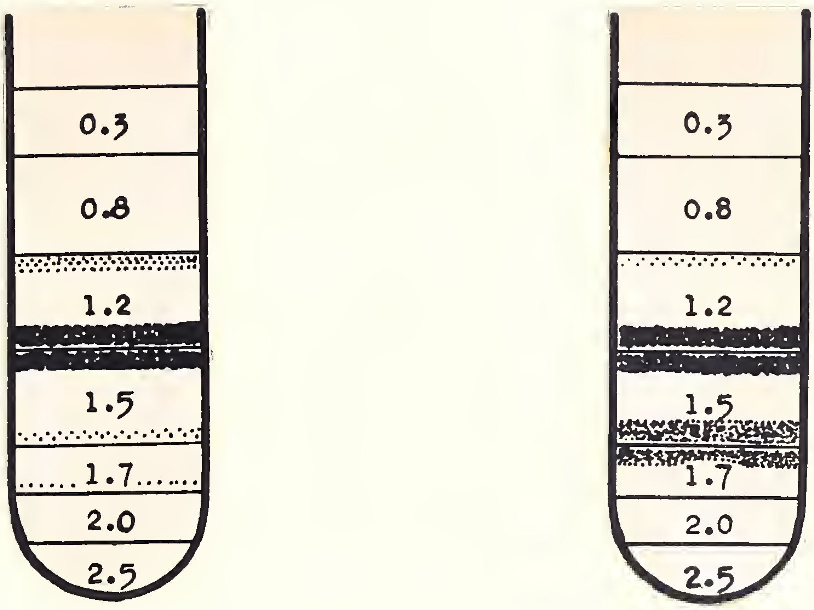


FIGURE 3. DISTRIBUTION OF 5-HT, HISTAMINE AND SUCCINIC OXIDASE (SO) IN THE DENSITY GRADIENT OF CELLS GROWN IN CULTURE. Arrows indicate where tube was cut. Dark horizontal lines indicate interfaces of the sucrose layers.

to the solid tumors, there was almost a complete lack of particulate material, including the non-sedimenting substance, above this band except for a very faint layer, F-2, at the 0.8 - 1.2 M interface. Another striking difference between the solid tumor and culture cells was the existence of a single, dense, layer of particulate material, F-4, at the 1.5 - 1.7 M interface instead of the two faint bands seen at this density in the experiments with the solid tumor (Fig. 2).

Cells grown in culture differed from those in the mouse not only in the distribution of the particulate material but also in the distribution of the amines. The dark, heavy band at the 1.2 - 1.5 M interface represents the mitochondria as indicated by the high percentage (60 per cent) of succinic oxidase in F-3 (Fig. 3). From 82 to 93 per cent of the amines were found in or below the mitochondrial band; less than 18 per cent of the amines were above the mitochondria. Another striking difference between these cells and the solid tumor was the high concentration (about 45 per cent) of the 5-HT and histamine that was located in the mitochondrial fraction.

Despite the fact that all experiments with cells obtained from culture were carried out with identical protocols, the distribution of particulate material in the density gradient varied. Two types of distribution were seen, and are depicted in Fig. 4. The consistently appearing, opaque layer, F-3, at the 1.2 - 1.5 M interface is present. However, the discrete,



(a) (b)

exp.	µg amine per mg cell	total µg in density gradient	exp.	µg amine per mg cell	total µg in density gradient
20	0.038 5-HT	0.00			
40	0.070 5-HT	0.00	37	0.23 5-HT	31.00
42	---- ----	----			
			45	--- Histamine	4.48
43	---- ---	----			
44	0.002 5-HT	0.00			

FIGURE 4. DENSITY GRADIENTS OF CELLS IN CULTURE. a) cells with negligible amounts of amines; b) cells with significant amounts of amines.

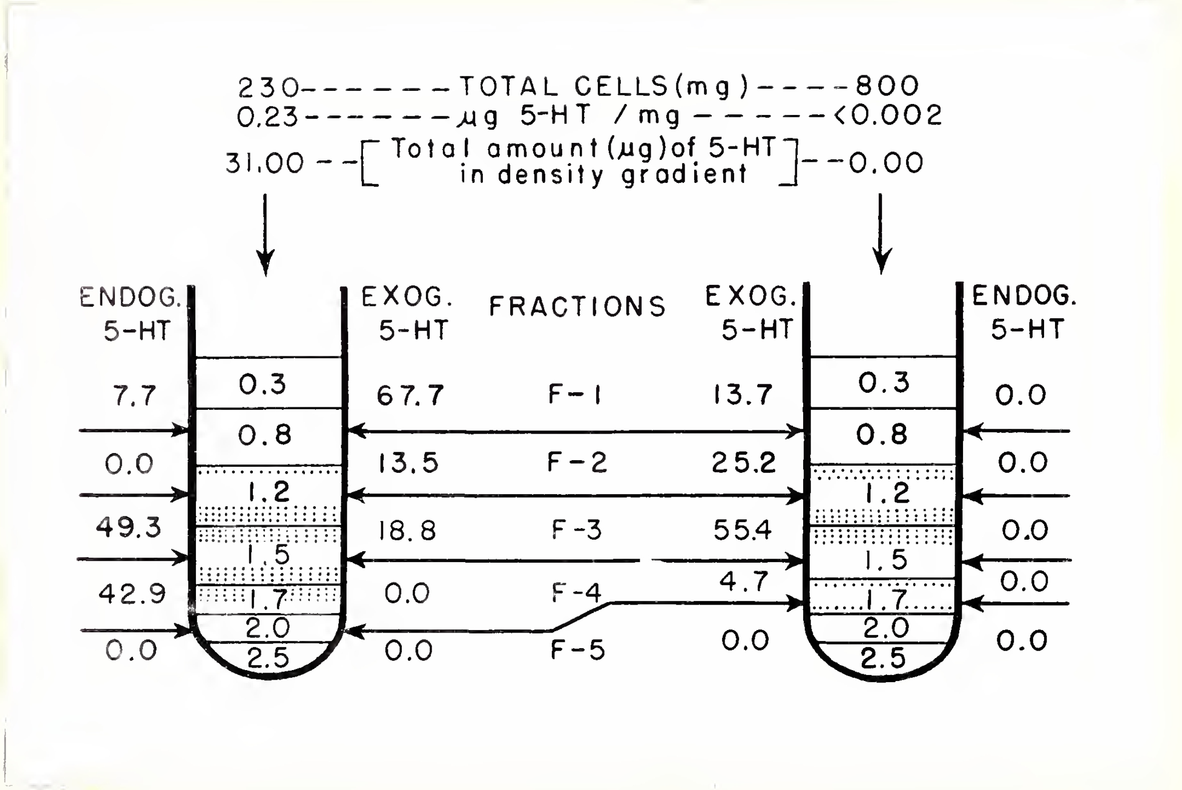
lower band that is present in Fig. 4b (and in Fig. 3) was absent in the experiments shown in Fig. 4a. In its place were two faint layers of material. Also, Fig. 4a shows an increase in the amount of material contained in the F-2 layer. These differences in the distribution of the particulate material were reflected in the differences in the total content of amines in the cells. Thus, those cells containing negligible amounts of particulate material at F-4 also contained negligible amounts of amines (_cf_. Fig. 4a and 4b). Although low in amines, the cells in the experiments represented in Fig. 4a contained mitochondria in the F-3 layer, as indicated by the high percentage of succinic oxidase activity in this fraction (Table 4).

B. _Intracellular Distribution of Exogenous Amines_

In four of the experiments on cells in culture (_viz_, exp. 37, 43, 44 and 45) the distribution of exogenous and endogenous amines was compared. These are illustrated in Fig. 5 and 6. It is apparent that the location of the _exogenous_ amines in the density gradient differed markedly from that of the _endogenous_ amines. Thus, 81.2 per cent of the exogenous 5-HT was located in fractions less dense than mitochondria (F-3), whereas 92.2 per cent of the endogenous amine was located in fractions as dense or denser than mitochondria. Similarily, 62.1 per cent of the exogenous histamine was found in fractions less dense than mitochondria, as contrasted with the location of 82.2 per cent

Fractions	exp. 40 (per cent)
F-1	
5-HT	0.0
SO	23.0
F-2	
5-HT	0.0
SO	0.0
F-3	
5-HT	0.0
SO	77.0
F-4	
5-HT	0.0
SO	0.0
F-5	
5-HT	0.0
SO	0.0
F-6	
5-HT	0.0
SO	0.0

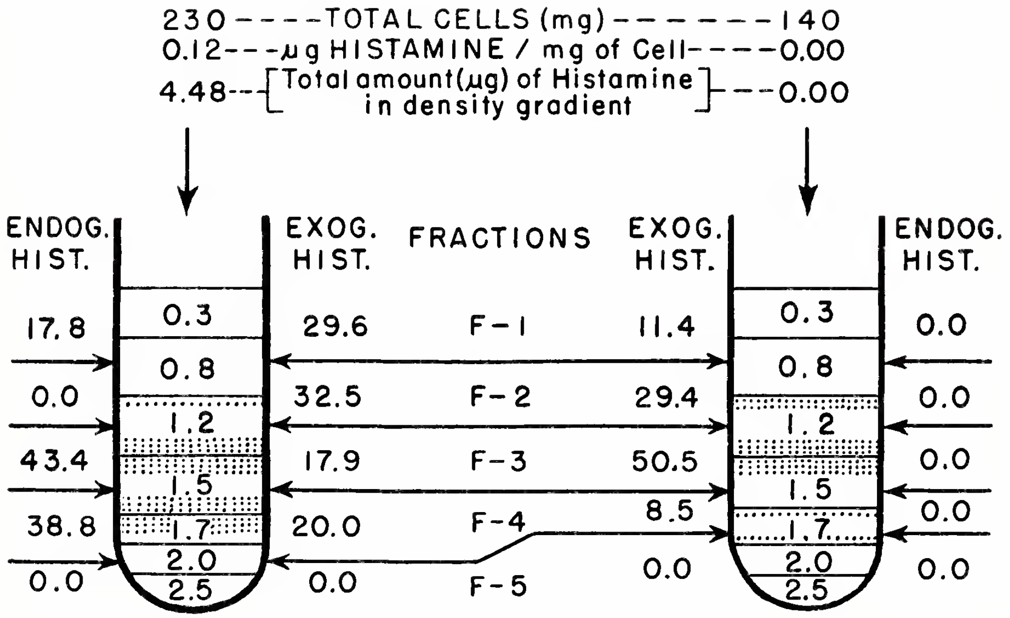
TABLE 4. DISTRIBUTION OF SUCCINIC OXIDASE (SO) AND 5-HT IN FRACTIONS AFTER DENSITY GRADIENT CENTRIFUGATION OF CULTURE CELLS CONTAINING NEGLIGIBLE AMOUNTS OF ENDOGENOUS AMINE



exp. 37

exp. 44

FIGURE 5. DISTRIBUTION OF ENDOGENOUS AND EXOGENOUS 5-HT IN CELLS IN CULTURE



exp. 45

exp. 43

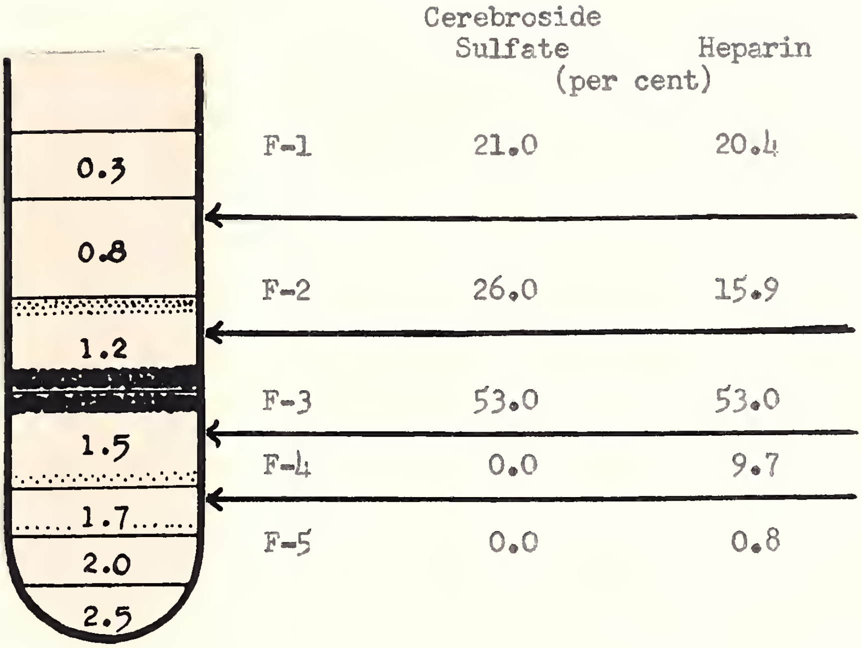
FIGURE 6. DISTRIBUTION OF ENDOGENOUS AND EXOGENOUS HISTAMINE IN CELLS IN CULTURE

of endogenous histamine in the mitochondrial or denser fractions. In these experiments, the cells contained significant amounts of endogenous amines. However, in cells containing extremely low levels of endogenous amines, the exogenous amines were distributed in a different manner. Thus, 50 per cent of the total exogenous amines were located in F-3 (mitochondria) with about 39 per cent distributed among F-1 and F-2.

C. Intracellular Distribution of Heparin and Cerebroside Sulfate in Cells in Culture

In one of the experiments with culture cells (exp. 42, Fig. 4a) the distribution of cerebroside sulfate and heparin in the density gradient was determined. The results of this experiment are given in Fig. 7 where it may be seen that the appearance of the gradient was consistent with those produced by cells poor in endogenous amines. (Fig. 4a).

The distribution of heparin and cerebroside sulfate was very similar. Also their distribution corresponded closely to that of the exogenous amines measured in cells that were also poor in endogenous 5-HT and histamine (see Fig. 5 and 6). Thus, about 50 per cent of the heparin, cerebroside sulfate and exogenous 5-HT and histamine were found in F-3 (mitochondria) and the remainder of these substances were distributed in a roughly parallel manner among F-1 and F-2.



	Cerebroside Sulfate	Heparin
	(per cent)	
F-1	21.0	20.4
F-2	26.0	15.9
F-3	53.0	53.0
F-4	0.0	9.7
F-5	0.0	0.8

FIGURE 7. DISTRIBUTION OF HEPARIN AND CEREBROSIDE SULFATE IN CULTURE CELLS CONTAINING NEGLIGIBLE LEVELS OF AMINES.

D. Intracellular Distribution of Phospholipid and Taurine in Solid Tumors

The distribution of phospholipid and taurine in solid tumors was different (Table 5). All of the measurable taurine was concentrated in F-1, which did not contain phospholipid; the lipid was distributed among the remaining amine-containing layers.

E. Intracellular Distribution of Ribonucleic Acid in Solid Tumors

Both the method of Schnieder and that of Kirby failed to detect ribonucleic acid in any of the amine-containing particulate material of solid tumors.

Fractions	exp. 23 (per cent)	exp. 24
F-1		
Histamine	20.9	30.9
SO	0.0	3.9
Taurine	----	100.0
Phospholipid	0.0	----
F-2		
Histamine	27.2	19.8
SO	7.3	8.1
Taurine	----	0.0
Phospholipid	19.0	----
F-3		
Histamine	14.6	16.5
SO	14.6	24.4
Taurine	----	0.0
Phospholipid	21.4	----
F-4		
Histamine	23.0	23.4
SO	57.4	46.2
Taurine	----	0.0
Phospholipid	38.0	----
F-5		
Histamine	3.9	3.7
SO	12.8	8.6
Taurine	----	0.0
Phospholipid	0.0	----
F-6		
Histamine	10.0	5.6
SO	7.8	8.5
Taurine	----	0.0
Phospholipid	15.7	----

TABLE 5. THE DISTRIBUTION OF TAURINE, PHOSPHOLIPID, HISTAMINE AND SUCCINIC OXIDASE (SO) IN FRACTIONS AFTER DENSITY GRADIENT CENTRIFUGATION OF THE SOLID MASTOCYTOMA

VI. DISCUSSION

A. Intracellular Distribution of Endogenous Amines

1. Solid Tumor

About 40 per cent of the histamine and 5-HT in this tumor was found in P-2, the large granular component. The rest of the amines were found in S-2, which contains both soluble material and microsomes (Table 1). This distribution was obtained in earlier work on this solid tumor (Green and Day, 1960).

Approximately one half of the amines in S-2 is bound to small particulate material of the density of microsomes (Green and Day, 1960). Some of the amines in the soluble material are almost certainly those lost from granules (Hagen *et al*, 1959). And another source of these amines may be exogenous amines (section VI, B).

The large granular component, P-2, when centrifuged in a density gradient, was resolved into several amine-containing

fractions (Fig. 2). Both the distribution of the particulate material and the distribution of the amines were the same, whether the tumor contained high (0.28 µg/g) or low (0.012 µg/g) levels of amines (Table 1).

The non-sedimenting fraction, F-1, which was contained entirely within the layer of 0.3 M sucrose had about 25 per cent of the amines in the large granular component. This fraction was cloudy but non-granular and was devoid of particles (Carlini and Green, 1962). It follows therefore that the amines here are in solution rather than bound to particulate material. The source of the amines in this fraction are probably two. Some were washed from the large granular component during resuspension. In fact, it has been shown that repeated washing of granular material with isotonic sucrose releases granular-bound amines (Hagen et al, 1959). Second, the amines in F-1 may represent those present in the soluble material that had been trapped in the interstices of the large granular component. As mentioned above some of the amines in the soluble material, and hence in F-1, may represent exogenous amines (section VI, B).

Fraction F-2, at the interface of 0.3-0.8 M sucrose, had about 20 per cent of the amines of the large granular component. This fraction has a density identical to that of microsomes (Carlini and Green, 1962); the microsomal fraction has previously been shown to be rich in these amines (Green and Day, 1960). No conclusion can be made as to whether the

amines in this fraction are contained in exceedingly small granules or are bound to the microsomes themselves.

The particles in the F-3 layer (0.8-1.2 M sucrose), which lies between the microsomes and mitochondria (Fig. 2), contained about 20 per cent of the amines (Table 2). This layer was separated into two by the interposition of 1.0 M sucrose, thereby indicating that the particulate material here is of at least two different densities. The mitochondrial fraction, F-4 (1.2-1.5 M sucrose) contained 20-30 per cent of the 5-HT and histamine present in the large granular component.

It is thus apparent that approximately 85 per cent of the total histamine and 5-HT present in the large granular component was in material (F-1, F-2, F-3, and F-4) either above or within the mitochondrial fraction; of this portion, 60 per cent was found in particulate material (F-2, F-3, and F-4). Only 15 per cent of the amines in the large granular component was found in fractions (F-5 and F-6) denser than mitochondria.

These results differ from findings with another mastocytoma (Furth) grown in the mouse (Hagen et al, 1959). In this tumor, 70 per cent of the amines were found in the large granular component (cf. 40 per cent in Table 1). Of this, 80 per cent were present in fractions denser than mitochondria in contrast to the X-1 tumor where negligible

amounts (15 per cent) were found in these fractions. (Mitochondria from both tumors had the same density.) Again unlike the X-1 tumor, which had amine-containing particles of widely varying density, almost all of the amines in the Furth tumor were located in one dense particulate fraction. Consonant with the homogeneity of the amine particles, electronmicrographs of the whole Furth tumor showed large granules of approximately the same size.

The differences between the distribution of amines in the X-1 tumor and in the Furth tumor are not necessarily contradictory, for comparable differences in the densities of amine-containing particles have been described before. Thus, the 5-HT-containing particles in brain (Whittaker, 1959) are less dense than those from the duodenum (Prusoff, 1960). The catecholamine-containing granules of the adrenal medulla (Blaschko _et al_, 1957) are denser than those from brain (Chruściel, 1960) and from adrenergic nerves (von Euler, 1958). And in brain, most of the histamine is found in the microsomal fraction (Carlini & Green, 1962), thus differing from mast cells.

The finding that the X-1 tumor has amine-containing granules with a range of densities is not without precedent. For example, catecholamine-containing granules

in adrenergic nerves vary in density from 0.8 to 1.2 M sucrose (von Euler, 1960), and in the brain 5-HT is found in the microsomal fraction as well as in denser particulate material (Carlini and Green, 1962). The fact that the X-1 tumor had, in contrast with the Furth tumor, large amounts of amines in the soluble material also has a parallel in observations on the distribution of 3-hydroxytyramine which is found in the soluble portion of sympathetic nerves (von Euler, 1958) but in the particulate material of the adrenal medulla (Eade, 1958).

Whether the 5-HT and histamine in the X-1 tumor are contained in the same granules is not known. On the basis of differing densities, separate 5-HT- and histamine-containing particles have been isolated from brain (Carlini and Green, 1962) and separate epinephrine and norepinephrine-containing particles have been isolated from adrenal medulla (Eade, 1958; Schümann, 1957). In the X-1 tumor, the consistent association of 5-HT and histamine in particles of a wide range of densities show that these amines are bound to particles of the same density. But the amines may not be in the same particle, for in brain the 5-HT- and acetylcholine-containing granules have the same density (Whittaker, 1959) but since these substances are not even found in the same cells (Paton, 1958), they cannot be present in the same granule. With regard to the X-1 tumor, all that one can conclude is that the granules

containing 5-HT and histamine are in the same cell, since the tumor arose from a single cell, and that these granules are of the same density.

2. Cells in Culture

Almost 95 per cent of the 5-HT in the X-1 cells grown in culture sedimented with the large granular component whereas only 40 per cent of the amines in the X-1 cells which were grown in the mouse sedimented with this fraction (*cf.* Table 1 and Table 3). Thus, the amine-containing material of culture cells, like that of the Furth solid tumor (Hagen *et al*, 1959), was denser than the amine-containing particles of the X-1 solid tumor. The large granular component of the culture cells, again like that of the Furth tumor, when centrifuged in a density gradient, resolved into only two distinct bands--the mitochondrial layer and one denser layer; together, these layers accounted for about 85 per cent of the amines present in the density gradient (Fig. 3). This finding contrasts with identical experiments on the X-1 solid tumor in which the large granular component showed three bands of particulate material--the mitochondrial layer and several *less* dense layers (Fig. 2); these layers accounted for 65 per cent of the 5-HT and histamine in the density gradient. Another difference between the culture cells and those grown in the mouse was the nearly complete absence of the dense particles in culture cells that contained low (0.07 µg/mg) amounts of amines, whereas the distribution of

particulate material from the cells grown in mice did not differ despite the occurrence, in some tumors, of similarly low (0.012 µg/mg) levels of amines. The impossibility of evaluating or even enumerating the vast number of variations existing between the *in vivo* and *in vitro* environments precludes any ready explanation for these differences.

On the other hand, some of the disparities that were observed between the X-1 cells grown in culture and the cells of both the X-1 solid tumor and the Furth solid tumor grown in the mouse may be explicable. Thus, the mitochondrial fraction of the culture cells contained more than one-half of the particulate-bound amines (Fig. 3) whereas the mitochondrial fraction of both the X-1 solid tumor (Table 2) and the Furth solid tumor (Hagen *et al*, 1959) contained not more than 30 per cent of the amines associated with particulate material. The presence of so great a percentage of 5-HT and histamine in the mitochondrial fraction of culture cells may be explained by postulating the presence in these cells of amine-containing granules that have the same specific gravity as mitochondria and hence are inseparable from them by this method. Alternatively it may be proposed that the amines present in the mitochondrial fraction of mast cells are not held in separate granules but are bound directly to the mitochondria. Obstensibly the low proportion of amines present in the

mitochondrial fraction from the solid tumors seems to rule out the proposition that mitochondria themselves store amines. However, it should be kept in mind that these solid tumors are contaminated by large numbers of connective tissue elements such as fibroblasts, histiocytes, and all of the cells that make up the blood vessels. Homogenates of such tissue would therefore contain the cytoplasm and mitochondria of these cells along with the cytoplasm, mitochondria and other particles of the mast cells. Thus, it follows that mast cell mitochondria, which may be rich in amines, will be diluted by a large number of amine-free mitochondria, and hence the percentage of 5-HT and histamine in the mitochondrial fractions of such preparations will appear spuriously lower than the true proportion of amines that may be associated with the mitochondria of the intact mast cell. The presence in mitochondria of substances capable of binding amines (see Green, 1962) is in accord with the hypothesis that mitochondria may in fact store amines.

A further difference between the cells grown in culture and those grown in the mouse was that the non-sedimenting material, F-1, in culture cells contained about 12 per cent of the amines in the large granular component (Fig. 3) compared with 25 per cent in the X-1 solid tumor (Table 2) and in the Furth solid tumor (Hagen

et al, 1959). If it is assumed that all of the amines present in F-1 are those that have been washed from the granules then one would have to presume that in cells from culture 5-HT and histamine are bound more strongly to their storage sites than are the amines of the solid tumor--an unlikely circumstance for it is reasonable to expect that the mechanism of binding 5-HT and histamine is the same in cells grown *in vivo* and *in vitro*. More likely, the relatively high proportion of amines in the F-1 layer of cells grown in the mouse are attributable to amines that have been taken up by the cells *in vivo*. That mast cells take up amines *in vivo* has been demonstrated (Day and Green, 1962), and evidence has been obtained (section VI, B) that such exogenous amines are present in the soluble material, part of which is found in F-1.

It should also be kept in mind that an even higher percentage of amines would be present in the F-1 fraction of the solid tumors if the soluble material that is present in this fraction had not been diluted by the amine-free solüble material of the contaminating connective tissue cells.

B. The Intracellular Distribution of Exogenous Amines

The location of most of the preformed 5-HT and histamine in fractions practically devoid of exogenous

amines support the proposition that there are at least two pools for amines. When cells containing endogenous amines were incubated with exogenous amines, about 72 per cent of the exogenous amines were found in fractions above the mitochondria, whereas about 86 per cent of the endogenous amines were found in the mitochondrial fraction (about 46 per cent) or in the dense particles below the mitochondria (about 40 per cent). When the same experiment was carried out on cells with negligible amounts of endogenous amines (the levels of amines in these cells fluctuate) the bulk of exogenous amines, about 52 per cent, was found in the mitochondrial fraction (Fig. 5 and 6). As noted before, cells low in exogenous amines had a dearth of particulate material below the mitochondria. It seems, therefore, that binding sites for endogenous amines, when not occupied by endogenous amines, may become available to exogenous amines. This idea is supported by the experiments shown in Fig. 5 and 6.

The amount of 5-HT present in density gradient in experiment 37 (see Fig. 5) was considerably higher than the level of histamine in the density gradient in experiment 45 (see Fig. 6). Therefore one would predict far fewer endogenous binding sites available for exogenous 5-HT in experiment 37 and a greater number of endogenous binding sites available for exogenous histamine in experiment

45. Thus, no exogenous 5-HT was associated with the dense amine-containing granules, most of it being located in the F-1 fraction, whereas 20 per cent of the exogenous histamine was associated with the amine-containing granules, along with a corresponding decrease in the proportion of this amine that was contained in the non-sedimenting fraction.

The existence of two pools for amines in these cells helps to explain some puzzling and divergent findings. The presence of exogenous amines in F-1 supports the idea that preformed exogenous amines contribute to the relatively high percentage of amines in the F-1 fraction when X-1 cells were grown in the mouse. Second, the fact that significant amounts of exogenous amines did not become bound to the mitochondrial fraction unless the cells were almost devoid of endogenous amines may indicate that endogenous amines are stored in this fraction, as well as in the fraction denser than mitochondria. The difference in turnover of endogenous and exogenous amines (Green and Day, 1962) is explicable on the basis of two pools for amines, one of which contains endogenous amines and is accessible to the exogenous amines, which are largely held in a separate pool.

C. Intracellular Distribution of Heparin and Cerebroside Sulfate in Cells in Culture

In cells with negligible amounts of endogenous amine, slightly more than 50 per cent of the heparin and

cerebroside sulfate was located in the mitochondrial fraction (F-3) with the rest distributed about equally in the two less dense fractions, F-1 and F-2 (Fig. 7). This distribution is almost identical to that of exogenous amines in cells that were similarily low in endogenous amines. Though these findings may be fortuitous, they are in agreement with the suggestion that heparin, and also possibly cerebroside sulfate, are important in the binding of amines by the mast cell. This parallel distribution of heparin, cerebroside sulfate and exogenous amines may further indicate that the same mechanisms that bind endogenous amines may also bind exogenous amines.

The high percentage of heparin found in the mitochondrial fraction of these mast cells in culture was not found in the Furth mastocytoma grown in the mouse (Hagen _et al_, 1959). These results are not necessarily contradictory when it is recalled that in studies with solid mastocytomas the mitochondria of connective tissue cells would dilute the mitochondria of the mast cells and yield spuriously low values for heparin in this fraction.

D. The Intracellular Distribution of Phospholipid and Taurine in Solid Tumors

The association of phospholipids in all of the amine-containing fractions of the X-1 solid tumor, except

the non-sedimenting layer, F-1, (Table 5) is consistent with the proposition that phospholipids play some role in amine binding by mast cells (see section II, B; also Green, 1962). Since the F-1 layer is composed of soluble material, it is not suprising that phospholipids, which are almost absent from the soluble material of other cells (Biezenski and Spaet, 1961), is absent from the F-1 layer. This may indicate that amines in the soluble material, most of which have been taken up by the cells from the mouse, are bound by a mechanism different from that in the particulate material of the cell. On the other hand taurine, which also has been implicated in amine-binding by mast cells (Green, 1962) was present only in F-1, but whether this finding implicates taurine in the binding of exogenous amines must await further studies.

E. Intracellular Distribution of Ribonucleic Acid in Solid Tumors

Ribonucleic acid could not be detected in the large granular component. If an acidic protein is present in significant quantity in the amine-containing material of mast cells, it is probably not ribonucleoprotein.

VII. SUMMARY

By density gradient centrifugation, the distribution of 5-HT and histamine in a mast cell tumor grown in culture differed from that of the same cells grown as a solid tumor. In culture, 85 per cent of the amine-containing particulate material was as dense or <u>denser</u> than the mitochondrial fraction whereas in solid tumors this amount of the amine-containing material was as dense or <u>less</u> dense than mitochondria.

Exogenous 5-HT and histamine were found in intracellular fractions different from these endogenous amines. But in cells containing insignificant levels of endogenous amines, the exogenous amines were found in sites normally occupied by endogenous amines. These observations are explicable on the basis of two pools for amines, one of which contains endogenous amines and is accessible to the exogenous amines, which are largely held in a separate pool.

In cells (grown in culture) containing insignificant levels of amines, heparin and cerebroside sulfate had an intracellular distribution similar to that of exogenous

amines. In solid tumors phospholipid was found in all the amine-containing fractions except the non-sedimenting material, which was rich in taurine. Ribonucleic acid could not be detected in any of the amine-containing material.

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